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# Rapid Method for Identification of Gram-Negative, Nonfermentative Bacilli

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A rapid system (OA), based on oxidative attack of substrates, was developed for identification of gram-negative, nonfermentative bacilli (NFB). One hundred and twelve strains of NFB from 25 species (representing the genera *Pseudomonas, Alcaligenes, Acinetobacter, Bordetella, Flavobacterium, Moraxella,* and *Xanthomonas*) were assayed by OA, buffered single substrate, and oxidative/fermentative methods. The 38 substrates consisted of salts of organic acids, nitrogen-containing compounds, alcohols, and carbohydrates. Ninety-four percent of the test strains were identified by the OA method in 24 h, and 99% were identifiable in 48 h. Reproducibility was 99%. Correlation with buffered single substrate was 98% (all substrates) and 90% with the oxidative/fermentative method (carbohydrates only). Biochemical profiles of all strains are presented, as well as tables showing the most useful tests for identification.

Gram-negative, nonfermentative bacilli (NFB) are widely distributed in nature and have been isolated from soil, water, milk, cosmetics, medical devices, and disinfectants (6, 8, 14, 17, 31), as well as clinical specimens. Identification of these organisms in a diagnostic laboratory is frequently incomplete and unsatisfactory. A common procedure is simply to report "*Pseudomonas* sp., *Alcaligenes* sp.," etc. The most commonly used tests are designed for the characterization of the *Enterobacteriaceae* and are frequently not applicable to those NFB which attack carbohydrates weakly or not at all. It has been shown by Park (20) and by Snell and Lapage (26) that oxidation of carbohydrates to acids by NFB in the presence of peptone can occur without any visible change of the indicator in the medium. Assimilation studies at the University of California have provided detailed taxonomic characterization of many of the NFB (1-4, 19, 24, 27). Although a few reports using this procedure have appeared in the literature (10-13), this method is not readily applicable in diagnostic bacteriology (25, 28). The buffered single substrate (BSS) system of Pickett and Pedersen (21-23) is very sensitive and can be used for many types of substrates. However, it is tedious to perform, and weakly positive reactions are difficult to read.

We report here an oxidative system for detecting microbial attack of various types of substrates (OA). It incorporates features from both the BSS and assimilation methods. When the OA system is used in conjunction with a small battery of conventional screening tests, most NFB can easily and rapidly be identified. The OA system was compared with the BSS and oxidative/fermentative (O/F) (16) methods in concurrent assays on 112 strains from 25 species of NFB. The genera examined included *Pseudomonas, Acinetobacter, Alcaligenes, Bordetella, Flavobacterium, Moraxella,* and *Xanthomonas.*

## MATERIALS AND METHODS

**Cultures.** Single strains of the following species were obtained from R. E. Weaver, Center for Disease Control, Atlanta, Ga.: *Pseudomonas maltophilia, P. stutzeri, P. putida, P. fluorescens, Bordetella bronchiseptica, Flavobacterium meningosepticum,* and *Moraxella osloensis.* One strain of *M. osloensis* was received from S. D. Henriksen. All other strains came from our collection.

**Substrates.** The organic salts tested were: butyrate, *m*-, *o*-, and *p*-hydroxybutyrate, citrate, lactate, malonate, nicotinate, propionate, saccharate, and tartrate. The amides and other nitrogen-containing compounds included acetamide, β-alanine, allantoin, asparagine, betaine, glutamine, nicotinamide, and phenylalanine. The battery of carbohydrates tested consisted of L-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, glycerol, *m*-inositol, lactose, maltose, D-mannitol, D-mannose, melibose, rhamnose, D-ribose, sorbitol, sucrose, D-trehalose, and D-xylose. The pH of the organic salts, amides, and nitrogen-containing compounds (hereafter, for convenience, referred to as "salts") was adjusted to 6.5 to 6.8 with 1 N HCl. Allantoin was adjusted to pH 9.0 since it is insoluble at an acid pH. All substrates were sterilized in concentrated solution (usually 10%) by being placed over chloroform for 48

h at room temperature. Salts were stored at room temperature, and carbohydrates were stored at 5 to 7 C.

**Alkalinization of salts.** The BSS method of Pickett and Pedersen (21) was used as a reference. Most of the substrates were tested at a concentration of 0.5%. Because of limited solubility, allantoin and asparagine were tested at 0.2% concentration and phenylalanine was tested at 0.25%.

The oxidative alkalinization medium (OAL) used with salts substrates consisted of (g/liter): $Na_2SO_4$, 2.0; $MgSO_4 \cdot 7H_2O$, 0.1; bromothymol blue, 0.08; yeast extract (Difco Laboratories, Detroit, Mich.), 0.5; Casamino Acids (Difco), 0.5; trace elements, 1.0 ml; $KH_2PO_4$, 4.539 (66.75 ml of 0.5 M solution); $K_2HPO_4$, 1.686 (19.37 ml of 0.5 M solution); Ionagar no. 2 (CoLab Laboratories, Inc., Glenwood, Ill.), 8.5. The trace elements solution contained, per liter of water: concentrated HCl, 12 ml; $CaCl_2 \cdot 2H_2O$, 100 mg; $CuSO_4 \cdot 5H_2O$, 394 mg; $MnCl \cdot H_2O$, 72 mg; $FeCl_3 \cdot 6H_2O$, 965 mg; $ZnSo_4 \cdot 7H_2O$, 8.840 g; $Na_2B_4O_7 \cdot 10H_2O$, 88.2 mg; $(NH_4)_2\ Mo_7O_{24} \cdot 4H_2O$, 36.8 mg. The compounds were added in the order given, mixing thoroughly after each addition. The medium was adjusted to pH 6.0 before autoclaving. After autoclaving, concentrated substrates were added to give a final concentration of 0.2%. An airtight, autoclavable, compartmented box was used for the OA system (Bionics Corp., Carson, Calif.). Each compartment contained a single substrate in 0.7 ml of basal medium. A single drop from a Pasteur pipette of a dense suspension (ca. $10^{11}$ organisms/ml) was inoculated on the agar surface of each compartment. The same suspension was used to inoculate the BSS medium. Daily readings were made for a maximum of 4 days. Strongly positive reactions were dark blue, weakly positive reactions were green, and negative reactions were yellow-green.

**Acidification of carbohydrates.** BSS (21) and O/F assays were performed concurrently on all strains. The O/F medium (Difco) was prepared according to the manufacturer's direction. The medium was inoculated by stabbing with a needle heavily loaded with cells from a 24-h slant. Only open tubes were used, with incubation for 7 days. The oxidative acidification basal medium (OAC) used in the OA system contained (g/liter): tryptone (Difco), 0.2; NaCl, 5.0; bromothymol blue, 0.08; $K_2HPO_4$, 0.304; Ionagar, 8.5. The pH was adjusted to 6.7 before autoclaving. All carbohydrates were added aseptically to give a final concentration of 2%. Inoculation was performed as described above. The OAC color reactions were bright yellow (in the medium) for a strongly positive reaction, green for a weakly positive reaction, and dark blue for a negative reaction.

Negative controls (inoculated basal media without added substrate) were included with all methods. A complete set of uninoculated substrates was included as a check on sterility and stability. All incubations were at 30 C as recommended by Stanier et al. (27).

After this work was completed, Ionagar no. 2 became unavailable. Chadwick et al. (7), in a similar situation, reported that OXOID agar no. 1 (Oxoid Ltd., London, England) proved equally satisfactory in identical concentrations.

## RESULTS AND DISCUSSION

The medium for carbohydrates (OAC) contains a small amount of tryptone (0.2 g/liter), which allows some growth of the NFB. A slight initial alkalinization occurs, followed (usually 1 day) by acidification. This medium can be used for nutritionally demanding organisms such as *P. maltophilia* (27). O/F medium (Difco) contains a higher concentration of peptone (2 g/liter) which can delay or entirely mask the appearance of a positive reaction (20, 26, 32). A comparison of reactions obtained on OA versus O/F is given in Table 1. Positive OA reactions occur earlier than those of O/F for *P. acidovorans* and *P. maltophilia*. *P. maltophilia* gave variable results in our hands on some substrates by OA, O/F, and BSS methods, with the O/F reactions generally delayed for 2 to 3 days. Fructose and maltose are useful in characterizing *P. maltophilia* when used in conjunction with alkali-producing substrates (see Table 9).

All organisms in this study were concurrently assayed by BSS on all substrates. Except for *Flavobacterium* sp. (see Table 3), the OA

TABLE 1. *Comparison of OA versus O/F reactions of weakly saccharolytic pseudomonads*

| Carbohydrates | *P. vesicularis* (3)[a] | | *P. acidovorans* (3) | | *P. maltophilia* (7) | | *P. pseudoalcaligenes* (4) | |
|---|---|---|---|---|---|---|---|---|
| | OA[b] | O/F[b] | OA | O/F | OA | O/F | OA | O/F |
| L-Arabinose | 2[2][c] | − | − | − | 3+ | + | − | − |
| Cellobiose | + | − | − | − | 4+ | $4+_w{}^3$ | − | − |
| D-Fructose | − | − | 2+ | +[4] | 1+ | $5+_w{}^3$ | + | + |
| D-Galactose | + | $+_w$ | − | 2+[4] | 5+[3] | $3+_w{}^2$ | − | − |
| D-Glucose | + | $+_w$ | − | − | +[2−3] | 3+[2] | − | − |
| Glycerol | − | − | 1+ | 1+[4] | − | − | 3+ | $1+_w{}^4$ |
| *m*-Inositol | − | − | − | 1+[4] | − | − | − | − |
| Lactose | − | − | − | − | 3+ | $2+_w{}^3$ | − | − |
| Maltose | + | $2+_w{}^2$ | − | − | + | $+_w{}^2$ | − | − |
| D-Mannitol | − | − | + | +[4] | − | − | − | 1+ |
| D-Mannose | − | − | − | − | 5+ | $5+_w{}^2$ | − | − |
| Melibiose | − | − | − | − | $3+_w{}^2$ | $3+_w{}^2$ | − | − |
| Rhamnose | − | − | − | − | − | − | − | − |
| D-Ribose | − | − | − | − | 1+ | − | − | − |
| Sorbitol | − | − | − | − | − | − | − | − |
| Sucrose | − | − | − | − | $3+_w{}^2$ | − | − | − |
| D-Trehalose | − | − | − | − | − | − | − | − |
| D-Xylose | 1+ | − | 1+ | − | $3+_w{}^2$ | − | − | − |

[a] Numbers in parentheses, number of strains.

[b] Abbreviations: OA, Oxidative acidification basal medium; O/F, oxidative/fermentative basal medium, open tube.

[c] +, All strains tested were positive within 1 day; −, all strains tested remained negative throughout the described period of incubation; $2+^2$, two of the strains tested gave positive results after 2 days of incubation; $4+_w{}^3$, four of the strains tested were weakly positive after 3 days of incubation.

system was equally or more sensitive than the BSS system for all genera. This sensitivity was indicated by the relative time for appearance of positive reactions. Table 2 shows the OA and BSS reactions of selected substrates for *P. acidovorans*, *P. maltophilia*, *P. pseudoalcaligenes*, and *P. stutzeri*. Note that *P. maltophilia* is readily distinguished from *P. pseudoalcaligenes* by the OA method, whereas they could be confused when tested by BSS. The increased sensitivity of OA salts (OAL) reactions is clearly demonstrated by the results obtained with *P. acidovorans*.

Although Table 3 shows that BSS is more sensitive than OA in detecting carbohydrate oxidation by flavobacteria (because of their highly proteolytic nature), the OA system still permits recognition of groups (see Tables 10 and 11). *F. meningosepticum* is distinguished by its attack of serine, fructose, glucose, glycerol, maltose, and mannitol; King's group IIB attacks citrate, allantoin, serine, and phenylalanine; group 3 attacks none of these substrates.

Stability studies on both OA media showed that prepared boxes stored at 5 to 7 C in sealed plastic bags are stable for at least 6 weeks. Ten carbohydrates (arabinose, cellobiose, fructose, inositol, lactose, mannitol, rhamnose, ribose, xylose, glycerol) were assayed weekly for 6 weeks with single strains of *P. putida* and *F.*

TABLE 2. *Comparison of OA versus BSS[a] reactions*

| Substrates | *P. acidovorans* (3)[b] | | *P. maltophilia* (7) | | *P. pseudoalcaligenes* (4) | | *P. stutzeri* (5) | |
|---|---|---|---|---|---|---|---|---|
| | OA | BSS | OA | BSS | OA | BSS | OA | BSS |
| Salts | | | | | | | | |
| Butyrate | + | − | 5+ | $+_w^3$ | $3+^{1-3}$ | $1+^3$ | $4+^2$ | 1+ |
| *p*-OH benzoate | + | − | − | − | − | − | − | − |
| Citrate | + | + | + | + | + | + | + | + |
| Lactate | + | − | + | + | + | + | + | + |
| Malonate | + | $+^2$ | 4+ | 5+ | − | − | + | + |
| Nicotinate | + | − | − | − | − | 1+ | 2+ | 1+ |
| Propionate | $+^2$ | − | + | $+^{1-3}$ | − | $1+_w^4$ | 3+ | 1+ |
| β-Alanine | $+_w$ | − | 1+ | − | + | $3+^3$ | 2+ | 1+ |
| Betaine | − | − | − | 1+ | $+^{1-3}$ | $1+_w^4$ | 1+ | 1+ |
| Serine | − | − | + | + | + | $+^3$ | + | 1+ |
| Carbohydrates | | | | | | | | |
| Cellobiose | − | $1+^3$ | 4+ | $6+_w^3$ | − | − | 1+ | 2+ |
| Fructose | 2+ | + | + | $+_w^3$ | + | 3+ | + | $+^{2-4}$ |
| Galactose | − | $+^{2-4}$ | $5+^3$ | $3+^2$ | − | − | + | $+^2$ |
| Glucose | − | − | $+^{2-3}$ | $5+_w^3$ | − | − | + | $+^2$ |
| Maltose | − | $2+^2$ | + | $+^2$ | − | − | 3+ | 2+ |
| Mannitol | + | + | − | − | − | − | 3+ | 3+ |
| Mannose | − | − | 5+ | $6+_w^3$ | − | − | + | $+^2$ |
| Xylose | 1+ | $1+^2$ | $3+_w^2$ | $4+_w^3$ | − | − | + | + |

[a] Abbreviations: BSS, Buffered single substrate; OA, oxidative acidification and oxidative alkalinization media; other abbreviations and notations as for Table 1.

[b] Numbers in parentheses, numbers of strains.

TABLE 3. *OA versus BSS reactions of Flavobacteria*

| Substrates | *F. meningosepticum* (5)[a] | | Group IIb[b] (4) | | Group 3[c] (4) | |
|---|---|---|---|---|---|---|
| | OA[d] | BSS | OA | BSS | OA | BSS |
| Salts | | | | | | |
| Butyrate | + | − | + | − | − | − |
| *m*-OH benzoate | − | − | − | − | − | − |
| *o*-OH benzoate | − | − | − | − | − | − |
| *p*-OH benzoate | − | − | − | − | − | − |
| Citrate | 2+ | 2+ | + | + | − | − |
| Lactate | 1+ | 1+ | − | − | − | − |
| Malonate | − | − | − | − | − | − |
| Nicotinate | − | − | − | − | − | − |
| Propionate | 1+ | 1+ | − | − | − | − |
| Saccharate | − | − | − | − | − | − |
| Tartrate | − | − | − | − | − | − |
| Acetamide | − | − | − | − | − | − |
| Nicotinamide | − | − | − | − | − | − |
| β-Alanine | − | − | − | − | − | − |
| Allantoin | − | − | + | 2+ | − | − |
| Asparagine | + | + | + | + | $+^2$ | $+^{2-4}$ |
| Betaine | − | − | − | − | − | − |
| Glutamine | − | − | − | − | − | − |
| DL-Serine | + | + | + | $+^{2-6}$ | − | − |
| Phenylalanine | − | − | 3+ | ND | − | − |
| Carbohydrates | | | | | | |
| L-Arabinose | − | − | 1+ | 1+ | − | − |
| Cellobiose | 1+ | + | − | 2+ | − | − |
| D-Fructose | + | + | − | − | − | − |
| D-Galactose | − | + | − | + | − | − |
| D-Glucose | + | + | − | + | − | − |
| Glycerol | 4+ | 4+ | − | − | − | − |
| *m*-Inositol | − | − | − | − | − | − |
| Lactose | 1+ | + | − | − | − | − |
| Maltose | + | + | 1+ | + | − | − |
| D-Mannitol | 4+ | 4+ | − | − | − | − |
| D-Mannose | + | + | 2+ | + | − | − |
| Melibiose | − | 4+ | ND | + | − | − |
| Rhamnose | − | − | − | − | − | − |
| D-Ribose | − | − | − | − | − | − |
| Sorbitol | − | $4+^2$ | − | − | − | − |
| Sucrose | − | 1+ | − | − | − | − |
| D-Trehalose | 2+ | ND | − | ND | − | − |
| D-Xylose | − | 2+ | − | − | − | − |

[a] Numbers in parentheses, number of strains.

[b] King's group IIb.

[c] Group 3 of Pickett and Pedersen (21), which may correspond to Center for Disease Control's group II-F.

[d] ND, Not done; other abbreviations and notations as for Tables 1 and 2.

*meningosepticum*. Ten salts (acetamide, allantoin, asparagine, glutamine, nicotinamide, citrate, malonate, saccharate, tartrate, and phenylalanine) were tested intermittently over a storage period of 2 months. Fifteen strains of the following organisms were used in these assays: *P. aeruginosa*, *P. putida*, *P. stutzeri*, *P. fluorescens*, *P. maltophilia*, *A. anitratus*, and

*A. lwoffi*. The organisms were chosen to reflect both weak and strong attack of the various substrates so that any deterioration during storage could be readily detected; none was found.

Those substrates listed above were also used to determine reproducibility of results. The media were freshly prepared for each test set. Only five individual variations occurred in 550 tests with salts (Table 4), an error rate of 0.9%. With carbohydrates there were seven variations in 480 tests, an error rate of 1.5%.

Concurrent assays (BSS and OA) of 108 strains (2,160 tests) on salts substrates gave a correlation of 98.7%. This correlation was calculated from the number (1.25%) of OAL-negative, BSS-positive paired results. Four and seven-tenths percent of the reactions were OA positive and BSS negative, which we believe reflects the basic difference in the two methods. The OAL medium contains growth factors in the yeast extract and Casamino Acids which allow a small amount of growth. BSS is a nongrowth system that consists of buffer, indicator, and substrate. Fastidious organisms may require one or more growth factors, such as biotin, for enzyme systems to produce a detectable change in a substrate. If an organism is unable to synthesize a needed growth factor from endogenous material, no attack of the substrate will occur. If a growth factor is the rate-limiting component of the enzyme system involved, providing it exogenously would increase the rate of substrate degradation, with a concomitant earlier appearance of measurable end products. These hypotheses may be a partial explanation of the observed OAL-positive, BSS-negative data.

The OAC and BSS assays for carbohydrate acidification were compared with 63 strains of NFB in 1,134 individual assays. Five percent of the paired assays were OAC positive, BSS negative. Again, this difference was considered negligible for the reasons given above. A correlation of 97% was found between the OAC and BSS tests, based on a 2.3% incidence of OAC-negative, BSS-positive reactions. Since OAC has been shown not to be as sensitive as BSS in detecting carbohydrate oxidation by the flavobacteria, those data were omitted from the calculations. Correlation of O/F with OAC reactions was 90%. All differences were due to failure of O/F to show any acidification after incubation for 7 days.

Although reaction times vary with the substrate and the organism, more than 95% of the OAL (salts) reactions were completed in one day versus 91% in BSS (Table 5). Ninety-nine percent were completed in 2 days on OAL versus 97% for BSS. No new positives appeared on OAL when incubation time was extended beyond 3 days. The time for positive reactions to appear on carbohydrates was longer for all three media. At 1 day, OAC had 91%, BSS had 84%, and O/F had 76% of all reactions completed. By day 2, OAC showed 98% of all reactions completed, whereas BSS and O/F had 93 and 92%, respectively. For carbohydrates, as for salts, no new positive reactions occurred when incubation was extended beyond three days.

When 13 species (single strains) were tested

TABLE 4. *Reproducibility of the OA system*

| Substrates | Organisms | Strain no. | No. of runs | Variations |
|---|---|---|---|---|
| Salts[a,b] | *P. putida* | 5 | 7 | None |
| | *P. fluorescens*[c] | 6 | 7 | 1 (tar +) |
| | *P. aeruginosa* | 128 | 11 | None |
| | *A. anitratus* | 133 | 11 | None |
| | *F. meningosepticum* | 26 | 7 | 1 ($\phi$ ala + $_w^2$) |
| | *P. pseudoalcaligenes* | 92 | 6 | 3 (2 nic +$_w^2$, $\phi$-ala +) |
| | *P. stutzeri* | 123 | 2 | None |
| | *P. maltophilia* | 122 | 2 | None |
| | *A. lwoffi* | 44 | 2 | None |
| Carbohydrates[a] | *P. putida* | 5 | 11 | 1 (lac −) |
| | *P. fluorescens* | 6 | 11 | None |
| | *P. aeruginosa* | 128 | 7 | 1 (cel −) |
| | *A. anitratus* | 133 | 6 | 2 (mannitol +) |
| | *F. meningosepticum* | 26 | 7 | 3 (2 ara +, lac −) |
| | *P. pseudoalcaligenes* | 92 | 6 | None |

[a] Ten salts and 10 carbohydrates were tested with each strain.

[b] Salts include the substrates which produce an alkaline reaction when degraded (see text).

[c] Abbreviations: tar, Tartrate; $\phi$ ala, phenylalanine; nic, nocotinamide; lac, lactose; cel, cellobiose; ara, L-arabinose; other abbreviations and notations as for Tables 1 and 2.

on 40 substrates (salts and carbohydrates) at 30 and 35 C, identical results were obtained in most instances. Twenty-one negative or delayed positive reactions were obtained at 35 C as compared with 30 C. Eleven instances of negative or delayed positive reactions occurred at 30 C versus 35 C.

TABLE 5. *Reaction rate for identification*

| Salts | | | Incubation days | Carbohydrates | | |
|---|---|---|---|---|---|---|
| OA | BSS | O/F | | OA | BSS | O/F |
| 96%[a] | 91% | NA[b] | 1 | 91% | 84% | 76% |
| 99% | 97% | NA | 2 | 98% | 93% | 92% |
| 100% | 99% | NA | 3 | 100% | 98% | 97% |
| | 100% | NA | 3+ | | 100% | 100% |

[a] Indicates percent reactions completed.
[b] NA, Not applicable.

The complete OA data on weakly saccharolytic NFB are given in Table 6. *M. phenylpyruvica* is separated from *M. osloensis* and *M. nonliquefaciens* on the basis of citrate and asparagine oxidation, as well as by the phenylpyruvic acid assay (30). On the basis of these and other studies (unpublished data), we doubt that there is any practical way to distinguish between *M. nonliquefaciens* and *M. osloensis* presently available.

Table 7 shows the reactions for nonsaccharolytic NFB. *Alcaligenes* sp. and *Bordetella bronchiseptica* attack many more substrates than do the two pseudomonads. In our system sac-

TABLE 6. *Moraxellae and weakly saccharolytic NFB: OA biochemical profiles*[a]

| Substrates | *P. acidovorans* (3) | *P. pseudoalcaligenes* (4) | *P. maltophilia* (7) | *P. vesicularis* (3) | *M. nonliquefaciens* (4) | *M. osloensis* (4) | *M. phenylpyruvica* (4) |
|---|---|---|---|---|---|---|---|
| Salts | | | | | | | |
| Butyrate | + | 3+[1-3] | 5+ | − | 1+ | 1+ | 3+ |
| *m*-OH benzoate | − | − | − | − | − | − | − |
| *o*-OH benzoate | − | − | − | − | − | − | − |
| *p*-OH benzoate | + | − | − | − | − | − | − |
| Citrate | + | + | + | − | − | 1+ | + |
| Lactate | + | + | + | − | + | + | + |
| Malonate | + | − | 5+ | − | − | − | − |
| Nicotinate | + | − | − | − | − | − | − |
| Propionate | +[2] | − | 6+ | − | 1+ | 3+ | + |
| Saccharate | + | − | − | − | − | − | − |
| Tartrate | + | − | − | − | − | − | − |
| Acetamide | + | − | − | − | − | − | − |
| Nicotinamide | + | − | − | − | − | − | − |
| β-Alanine | +w | + | 1+ | − | − | − | − |
| Allantoin | + | 3+[2] | − | − | − | − | − |
| Asparagine | + | + | + | + | − | − | + |
| Betaine | − | +[1-3] | − | − | − | − | − |
| Glutamine | + | + | 2+ | − | − | − | − |
| DL-Serine | − | + | + | − | − | − | − |
| Phenylalanine | ND | 1+ | − | − | − | − | − |
| Carbohydrates | | | | | | | |
| L-Arabinose | − | − | 3+ | 2+[2] | − | 3+[2] | − |
| Cellobiose | − | − | 4+ | + | − | − | − |
| D-Fructose | 2+ | + | + | − | − | − | − |
| D-Galactose | − | − | 5+[3] | + | − | − | − |
| D-Glucose | − | − | +[2-3] | + | − | − | − |
| Glycerol | 1+ | 3+ | − | − | − | − | − |
| *m*-Inositol | − | − | − | − | − | − | − |
| Lactose | − | − | 3+ | − | − | − | − |
| Maltose | − | − | + | + | − | − | − |
| D-Mannitol | + | − | − | − | − | − | − |
| D-Mannose | − | − | 5+ | − | − | − | − |
| Melibiose | − | − | 3+[2] | − | − | − | − |
| Rhamnose | − | − | − | − | − | − | − |
| D-Ribose | − | − | 1+ | − | − | 3+[2] | − |
| Sorbitol | − | − | − | − | − | − | − |
| Sucrose | − | − | 3+[2] | − | − | − | − |
| D-Trehalose | − | − | − | − | − | − | − |
| D-Xylose | 1+ | − | 3+[2] | + | − | 3+[2] | − |

[a] Abbrevations and notations as for Tables 1 and 2.

TABLE 7. *Nonsaccharolytic NFB: OA biochemical profiles*[a]

| Salts | *A. denitrificans* (3) | *A. faecalis* (4) | *A. odorans* (5) | *B. bronchiseptica* (2) | *A. lwoffi* (5) | *P. alcaligenes* (4) | *P. diminuta* (6) |
|---|---|---|---|---|---|---|---|
| Butyrate | 2+ | + | + | + | + | 3+ | 4+ |
| *m*-OH benzoate | − | − | − | − | − | − | − |
| *o*-OH benzoate | − | − | − | − | − | − | − |
| *p*-OH benzoate | − | − | − | − | − | − | − |
| Citrate | + | + | + | + | 4+ | 1+ | − |
| Lactate | + | + | + | + | 4+[2] | + | − |
| Malonate | + | +[2-3] | + | + | − | − | − |
| Nicotinate | 2+ | + | + | + | − | − | − |
| Propionate | 2+ | + | + | + | 4+ | 3+ | 1+ |
| Saccharate | + | + | − | + | − | − | − |
| Tartrate | + | − | − | 1+ | − | − | − |
| Acetamide | + | + | + | − | − | − | − |
| Nicotinamide | − | 2+ | +[1-3] | − | − | − | − |
| β-Alanine | + | + | 3+ | 1+ | 1+ | − | 4+ |
| Allantoin | 2+[2] | 3+ | 1+ | + | − | − | − |
| Asparagine | + | + | + | + | + | + | 5+ |
| Betaine | − | − | − | − | − | − | − |
| Glutamine | + | + | + | + | − | + | − |
| DL-Serine | + | + | + | + | 3+[2] | − | 5+ |
| Phenylalanine | 1+ | − | − | − | − | − | − |

[a] Abbreviations and notations as for Tables 1 and 2.

charate and tartrate are important substrates in distinguishing *A. odorans* from the other *Alcaligenes* sp. and *B. bronchiseptica*. *A. faecalis* can be distinguished from *B. bronchiseptica* by its pattern of attack of acetamide and β-alanine. King (18) distinguished *B. bronchiseptica* from *Alcaligenes* sp. on the basis of urease. The three *Alcaligenes* sp. are separated by their attack of nitrate and nitrite (18, 21) and the pattern of attack of organic salts and amides (21). An occasional strain of *A. lwoffi* is weakly oxidase positive, a misleading reaction, which would indicate that the strain is a member of the genus *Moraxella*. Aside from penicillin sensitivity, the only test to distinguish such strains from *M. phenylpyruvica* is the phenylpyruvic acid assay.

The saccharolytic NFB (Table 8) extensively attack both carbohydrates and salts. The acidification of some carbohydrates (arabinose, cellobiose, galactose, glucose, mannose, maltose, lactose, ribose, and xylose) by *A. anitratus* is caused by a nonspecific aldose dehydrogenase, which oxidizes them to their corresponding sugar acids (4, 15). Thus, only one sugar need by assayed to separate *A. anitratus* from *A. lwoffi*.

Detailed analysis of the reactions of individual strains (except *Moraxella* strains) used in our study showed that 94.7% could be identified after 24 h of incubation. After 48 h of incubation, 100% could be identified to the species level. In no instance was incubation beyond 3 days either necessary or desirable. This analysis took into account only those substrates recommended in Tables 9 through 11. For convenience in identification, "conventional" test data from Pickett and Pedersen (22, 23) were included in these tables. Most of these tests were performed at 35 C. Gilardi (13) reported on six genera of NFB isolated from clinical specimens. Eight species accounted for 80% of the strains reported; these were: *A. anitratus*, *P. maltophilia*, *P. putida*, *A. lwoffi*, apyocyanogenic *P. aeruginosa*, saccharolytic *A. haemolyticus*, *P. stutzeri*, and *P. fluorescens* (in descending order of frequency). Two species, *A. anitratus* and *P. maltophilia*, accounted for greater than 50% of the strains reported. Others (5, 9, 29) have reported that *P. aeruginosa* is the most frequently encountered NFB in hospital laboratories. Our strains of *A. anitratus* and *P. maltophilia* were identifiable at the end of 24 h of incubation. Of the eight most common species listed by Gilardi, our schema identified 98% in 24 h. One strain of *P. fluorescens* required a second day of incubation before positive identification could be made. The more rarely encountered NFB listed were all identifiable in 2 days, with 95% of all strains characterized after 24 h of incubation. Those species identified after 24 h of incubation were: *A. odorans*, *M. nonliquefaciens*, *F. meningosepticum*, *P. alcaligenes*, *P. acidovorans*, *P. pseudoalcaligenes*, *A. faecalis*, *M. osloensis*, *A. denitrificans*, *B. bronchiseptica*, *M. phenylpyruvica*, three of

TABLE 8. *Saccharolytic NFB: OA biochemical profiles*[a]

| Substrates | *P. aerugi-nosa* (5) | *P. fluores-cens* (5) | *P. putida* (5) | *P. cepacia* (5) | *P. picket-tii* (7) | *P. stutzeri* (5) | *A. anitra-tus* (6) | *Xantho-monas* (5) |
|---|---|---|---|---|---|---|---|---|
| Salts | | | | | | | | |
| Butyrate | 4+ | + | + | 4+ | +[1-3] | 4+ | + | − |
| *m*-OH benzoate | − | − | − | − | − | − | − | − |
| *o*-OH benzoate | − | − | − | − | − | − | − | − |
| *p*-OH benzoate | 3+ | + | + | + | 3+ | − | 5+ | 3+ |
| Citrate | + | + | + | + | 6+ | + | + | 3+ |
| Lactate | + | + | + | + | + | + | 4+ | + |
| Malonate | + | + | 1+ | + | 5+ | + | − | − |
| Nicotinate | − | 2+ | + | 4+ | − | 2+ | − | − |
| Propionate | 4+ | 4+ | + | + | 6+ | 3+ | + | − |
| Saccharate | − | 4+ | + | + | 5+ | + | − | − |
| Tartrate | − | − | + | + | 5+ | 2+ | − | − |
| Acetamide | + | − | − | 3+ | − | 1+ | − | − |
| Nicotinamide | − | − | 1+ | 2+ | − | − | − | − |
| β-Alanine | + | + | + | + | + | 2+ | 3+ | − |
| Allantoin | + | 4+ | + | + | + | 2+ | 4+ | − |
| Asparagine | + | + | + | + | + | + | + | + |
| Betaine | + | + | + | 4+ | 2+ | 1+ | − | 4+ |
| Glutamine | 4+ | + | + | + | 6+ | + | 3+ | − |
| DL-Serine | 4+ | 4+ | + | 4+ | 5+ | +[2] | 5+ | 4+ |
| Phenylalanine | − | − | − | ND | − | − | − | − |
| Carbohydrates | | | | | | | | |
| L-Arabinose | + | + | + | + | 6+ | + | + | + |
| Cellobiose | 4+[2-3] | +[1-3] | +[1-3] | + | 6+ | 1+ | + | + |
| D-Fructose | + | + | + | + | 3+ | + | 1+ | + |
| D-Galactose | + | + | + | + | + | + | + | + |
| D-Glucose | + | + | + | + | + | + | + | + |
| Glycerol | + | + | + | 4+ | 6+ | + | 1+ | 1+ |
| *m*-Inositol | − | 2+ | − | + | 1+ | − | − | − |
| Lactose | 1+ | 4+[2-3] | +[2-3] | + | 5+[1-3] | 1+ | + | + |
| Maltose | − | − | − | + | 4+[1-3] | 3+ | 5+[3] | + |
| D-Mannitol | + | 4+ | − | + | − | 3+ | − | − |
| D-Mannose | 4+ | + | + | + | + | + | + | + |
| Melibiose | 4+ | + | + | − | 2+ | + | + | + |
| Rhamnose | 3+[2] | + | +[2] | − | 1+ | 2+[2] | + | 1+ |
| D-Ribose | + | + | + | +[2] | +[2-3] | + | + | − |
| Sorbitol | − | 1+ | − | + | − | − | − | − |
| Sucrose | − | 2+ | − | + | − | − | − | + |
| D-Trehalose | 2+ | 2+ | − | + | − | − | − | + |
| D-Xylose | + | + | + | + | + | + | + | + |

[a] Abbreviations and notations as for Tables 1 through 3.

five strains of *P. diminuta*, and four of five strains of *P. cepacia*. Our strains that were not listed by Gilardi were: *P. pickettii*, *P. vesicularis*, *Xanthomonas*, *Flavobacterium* groups IIb and 3. Ninety-one percent were identified in 24 h and 100% after 48 h of incubation. One strain each of *P. vesicularis* and *Flavobacterium* IIb required 48 h of incubation.

The OA system presents many advantages over other systems currently available. The principal advantage is the ease of inoculation. Multiple substrates can be inoculated in a short time. For example, we have inoculated over 400 substrates in 30 min. Other systems require removal and replacement of caps from individual tubes containing a single substrate. This contributes a substantial amount of time to the inoculation. In our system, lifting the cover exposes 10 substrates and two negative controls (basal medium without added substrate). We routinely inoculate only one control for comparison with positive reactions. The uninoculated control serves as a check for sterility and stability of basal medium.

Since the boxes are small (2 by 3 by 3/4 inches [ca. 5.08 by 7.62 by 1.92 cm]) and stackable, very little incubator space is needed. A small incubator will accommodate about 200 boxes. If 20 substrates are used (10 carbohydrates and 10 salts), two boxes will be used for each isolate. Under these conditions, 100 strains could be assayed in an incubator with interior dimen-

TABLE 9. *Saccharolytic pseudomonads: recommended tests*

| Test | Characteristics | *P. aerugi-nosa* (26) | *P. fluo-rescens* (15) | *P. putida* (6) | *P. cepa-cia* (7) | *P. picket-tii* (22) | *P. stutz-eri* (13) | *P. malto-philia* (14) |
|---|---|---|---|---|---|---|---|---|
| Conventional[a] | Pigmented colony | 13+[b] | − | − | 2+ | 1+ | 2+ | 2+ |
| | Oxidase | + | + | + | + | + | + | 1+ |
| | Motility | + | 14+ | + | + | + | + | + |
| | $NO_3 \rightarrow NO_2$ | + | + | + | 3+ | + | + | 6+ |
| | $NO_2 \rightarrow N_2$ | + | 3+ | − | − | − | 9+ | − |
| | Fluorescence | 25+ | + | + | − | − | − | − |
| | 42 C growth | + | − | − | 4+ | 14+ | + | 4+ |
| | Indol | − | − | − | − | − | − | − |
| | Gluconate | 22+ | 13+ | + | − | − | − | − |
| | LDC | − | − | − | + | − | − | + |
| OA | | (5) | (5) | (5) | (5) | (7) | (5) | (7) |
| | Carbohydrates | | | | | | | |
| | Arabinose | + | + | + | + | 6+ | + | 3+ |
| | Cellobiose | 4+[2] | +[2] | +[2] | + | 6+ | 1+ | 4+ |
| | Fructose | + | + | + | + | 3+ | + | + |
| | Lactose | 1+ | 4+[2] | +[2] | + | 5+ | − | 3+ |
| | Maltose | − | − | − | + | 4+[1-3] | 3+ | + |
| | Mannitol | + | 4+ | − | + | − | 3+ | − |
| | Salts | | | | | | | |
| | Acetamide | + | − | − | 3+ | − | 1+ | − |
| | Allantoin | + | 4+ | + | + | + | 2+ | − |
| | Malonate | + | + | 1+ | + | 5+ | + | 5+ |
| | Propionate | 4+ | 4+ | + | + | 6+ | 3+ | 6+ |
| | Saccharate | − | 4+ | + | + | 5+ | + | − |
| | Tartrate | − | − | + | + | 5+ | 2+ | − |
| | Serine | 4+ | 4+ | + | 4+ | 5+ | +[2] | + |
| | β-Alanine | + | + | + | + | + | 2+ | 1+ |

[a] "Conventional tests" data are from Pickett and Pedersen (22, 23).
[b] Abbreviations and notations as for Tables 1 and 2.

sions of 13 by 13 by 13½ inches (ca. 33.02 by 33.02 by 34.29 cm).

Carbohydrates must be in a separate box from the salts because the volatile by-products of degradation by either type can cause false reactions. The tryptone in OAC is initially attacked by all of the organisms we tested with the production of ammonia. The ammonia volatilizes and is reabsorbed in all compartments, which then turn blue. If salts substrates are in the same box, they would show false positives (blue is the positive end point for salts). Conversely, if strong attack of a nitrogen-containing compound occurs in a box also containing carbohydrates, the ammonia produced by deamination would cause false-negative reactions for those carbohydrates which are weakly attacked. The small amount of acid normally produced would not be enough to cause a visible change in the indicator (20, 26). One of the reasons that OAC is more sensitive than O/F medium is that the smaller amount of tryptone (0.2 g versus 2 g per liter) in OAC results in less ammonia production and less masking of acid reactions.

Positive reactions appear relatively quickly (1 day) due to two factors: (i) the low buffering capacity of the media, and (ii) the use of large numbers of cells with preformed enzymes. Reactions are distinct and unambiguous. Bromothylmol blue is an intensely colored indicator, with a short pH range (6.0 to 7.6). Compartmentalization allows the use of an individual substrate in a small volume (0.5 to 0.7 ml). It also prevents interference by strong reactions as well as increasing the sensitivity of the assay due to increased color intensity.

We have noted several papers dealing with various attempts to adapt the basic concepts of the assimilation method for routine use (7, 25). Rosenthal (25) used substrate-impregnated disks applied to an inoculated basal medium and used an increased amount of growth around the disk as a positive reaction. Chadwick et al. (7) advocate the use of an antibiotic sensitivity replicator for multiple inoculation of agar plates, each containing a single substrate. These methods still have the disadvantages of requiring relatively large amounts of media and are easily subject to misinterpretation

TABLE 10. *Saccharolytic and weakly saccharolytic NFB: recommended tests*[a]

| Test | Characteristics | *Xanthomonas* (7) | *A. anitratus* (23) | *F. meningosepticum* (1) | *P. acidovorans* (15) | *P. pseudoalcaligenes* (8) | *P. vesicularis* (1) |
|---|---|---|---|---|---|---|---|
| Conventional | Pigmented colony | + | − | + | − | − | − |
| | Oxidase | + | − | + | + | + | + |
| | Motility | + | − | − | + | + | + |
| | $NO_3 \rightarrow NO_2$ | − | − | − | + | + | − |
| | $NO_2 \rightarrow N_2$ | − | − | − | − | − | − |
| | Fluorescence | − | − | − | − | − | − |
| | 42 C growth | − | 22+ | − | − | 1+ | − |
| | Indol | − | − | + | − | − | − |
| | Gluconate | − | − | − | − | − | − |
| | LDC | − | − | − | − | − | − |
| OA | | (5) | (6) | (5) | (3) | (4) | (3) |
| | Carbohydrates | | | | | | |
| | Arabinose | + | + | − | − | − | 2+[2] |
| | Cellobiose | + | + | 1+ | − | − | + |
| | Fructose | + | 1+ | + | 2+ | + | − |
| | Lactose | + | + | − | − | − | − |
| | Maltose | + | 5+[3] | + | − | − | + |
| | Mannitol | − | − | 4+ | + | − | − |
| | Salts | | | | | | |
| | Acetamide | − | − | − | + | − | − |
| | Allantoin | − | 4+ | − | + | 3+[2] | − |
| | Malonate | − | − | − | + | − | − |
| | Propionate | − | + | 1+ | +[2] | − | − |
| | Saccharate | − | − | − | + | − | − |
| | Tartrate | − | − | − | + | − | − |
| | Serine | + | 4+ | + | − | + | − |
| | β-Alanine | − | 3+ | − | + | + | − |

[a] Soluble brown pigment is formed by *P. vesicularis* and sometimes by *P. pseudoalcaligenes*; other abbreviations and notations as for Table 9.

TABLE 11. *Moraxellae and nonsaccharolytic NFB: recommended tests*[a]

| Test | Characteristics | *P. diminuta* (6) | *P. alcaligenes* (12) | *A. denitrificans* (1) | *A. faecalis* (1) | *A. odorans* (7) | *B. bronchiseptica* (13) | *A. lwoffi* (16) | *Flavobacterium* group IIb (16) | *Flavobacterium* group 3 (8) | *M. nonliquefaciens* (14) | *M. osloensis* (4) | *M. phenylpyruvica* (4) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Conventional | Pigmented colony | 1+ | − | − | − | − | − | − | + | + | − | − | − |
| | Oxidase | + | + | + | + | + | + | − | + | 3+ | + | + | + |
| | Motility | + | + | + | + | + | + | − | − | − | − | − | − |
| | $NO_3 \rightarrow NO_2$ | − | + | + | − | − | 6+ | − | 5+ | − | 7+ | + | − |
| | $NO_2 \rightarrow N_2$ | − | − | − | − | + | − | − | − | − | − | − | − |
| | Fluorescence | − | − | − | − | − | − | − | − | − | − | − | − |
| | 42 C growth | 5+ | + | + | + | + | 12+ | 15+ | 3+ | + | 5+ | + | + |
| | Penicillin sensitive (2-unit disk) | − | − | − | − | − | − | − | − | + | + | + | + |
| | Indol | − | − | − | − | − | − | − | 15+ | + | − | − | − |
| | Gluconate | − | − | − | − | − | − | − | − | − | − | − | − |
| | LDC | − | − | − | − | − | − | − | − | − | − | − | − |
| | Additional features | | Gln+ | | | | Urea+ | | | Gel+ | | | Asn+ |
| OA | | (6) | (5) | (3) | (4) | (5) | (2) | (5) | (4) | (4) | (4) | (4) | (4) |
| | Salts | | | | | | | | | | | | |
| | Acetamide | − | − | + | + | + | − | − | − | − | − | − | − |
| | Allantoin | − | − | 2+[2] | 3+ | 1+ | + | − | + | − | − | − | − |
| | Malonate | − | − | + | + | + | + | − | − | − | − | − | − |
| | Propionate | 1+ | 3+ | 2+ | + | + | + | 4+ | − | − | 1+ | 3+ | + |
| | Saccharate | − | − | + | + | − | + | − | − | − | − | − | − |
| | Tartrate | − | − | + | − | − | 1+ | − | − | − | − | − | − |
| | Serine | 5+ | − | + | + | + | + | 3+ | + | − | − | − | − |
| | β-Alanine | 4+ | − | + | + | 3+ | 1+ | 1+ | − | − | | − | − |

[a] Gln, Glutamine; gel, gelatin; asn, asparagine; other abbreviations and notations as for Table 9.

when fastidious organisms are involved. We suggest that the OA system overcomes all of the disadvantages inherent in other systems and provides a useful tool for the identification of nonfermentative bacilli.

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